Tumoricidal Effects of Etoposide Incorporated Into Solid Lipid Nanoparticles After Intraperitoneal Administration in Dalton's Lymphoma Bearing Mice

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ABSTRACT

The tumoricidal effects of etoposide incorporated into lipid nanoparticles after single-dose administration were investigated in Dalton's lymphoma ascites bearing mice. Etoposide and its nanoparticle formulations were administered intraperitoneally, and the cell cycle perturbation, cytogenetic damage, cell death (apoptosis), tumor regression, and animal survival were investigated as parameters of response with time. The tumor burden of mice treated with etoposide and its nanoparticle formulations decreased significantly ($P < .001$) compared with the initial up to 4 to 6 days, followed by an increase at later time intervals. Of the 3 different formulations, the survival time of mice was higher when treated with etoposide-loaded tripalmitin (ETP) nanoparticles, followed by etoposide-loaded glycerol monostearate (EGMS) (27.3%) and etoposide-loaded glycerol distearate (EGDS) (27.3%) compared with free etoposide. Cell cycle analysis revealed the hypodiploid peak (sub G_0/G_1 cell population) as well as G_2 arrest in mice treated with etoposide and its nanoparticle formulations. The frequency of dead cells treated with the nanoparticle formulations remained high even after 8 days of treatment compared with free etoposide. The mice treated with nanoparticle formulations exhibited hypodiploid peaks and reduced S phase even 8 days after treatment, whereas the free etoposide-treated mice showed decrease in apoptosis after 3 days of treatment. The apoptotic frequency in cells 17 days after treatment was in the order of ETP > EGMS > EGDS > etoposide. The experimental results indicated that among the 3 nanoparticle formulations studied, the ETP nanoparticles showed greater and prolonged apoptotic induction properties, resulting in the higher increase in survival time of tumor bearing mice.

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INTRODUCTION

Despite advances in cancer chemotherapy, the treatment of peritoneal carcinomas remains a significant failure. Unlike solid tumors, which can be removed by surgery, the peritoneal tumors result in inoperable accumulation of malignant ascites, and hence to date their treatment remains a challenge. Peritoneal concentrations of intravenously administered drugs remain low owing to poor drug penetration into the peritoneal cavity and therefore would be insufficient to eliminate such tumors. Intraperitoneal chemotherapy of peritoneal tumors would likely be a better method owing to high local concentration in tumor regions and low systemic toxicity. However, effective prolonged treatment of tumors by conventional intraperitoneal chemotherapy with anticancer agents is doubtful because of their rapid elimination from the peritoneal cavity[1] and distribution to other organs, as well as their lower half-life and stability. Techniques such as chemotherapeutic peritoneal perfusion of anticancer agents have been used with little success,[2,3] and such techniques complicate the therapy by decreasing patient compliance. Theoretically the drawbacks associated with intraperitoneal chemotherapy of anticancer agents could be overcome by incorporation into drug delivery matrices, which facilitate sustained drug release for prolonged time periods. Such sustained release in peritoneal cavity would lead to enhanced cell exposure to anticancer agent for prolonged periods and would likely result in an effective cell kill. Tamura and coworkers[4] observed that, in peritoneal tumor bearing mice, the intraperitoneal administration of cisplatin in poly(D,L-lactic acid) microspheres induced sustained tumor growth inhibition along with prolonged survival time.

Nanoparticulate delivery systems have been widely investigated as drug carriers because of advantages such as smaller size, controlled drug release potential, targeting ability,

enhancement of therapeutic efficacy, and reduction of toxicity.[5,6] Lipid nanoparticles have recently received considerable attention as alternative drug carriers to emulsions, liposomes, and nanoparticles[7,8] The solid lipids used in nanoparticle preparation possess excellent stability, biocompatibility, biodegradation, and low cellular and systemic toxicity.[6] A striking advantage of lipid nanoparticles is the possibility of large-scale production by high pressure homogenization.[9] Their stability can be further improved by lyophilization[10] or spray drying.[11]

Etoposide is an anticancer agent used in the treatment of a variety of malignancies including malignant lymphomas.[12] It acts by inhibition of topoisomerase-II and activation of oxidation reduction reactions to produce derivatives that bind directly to DNA and cause DNA damage.[13,14] The effective chemotherapy of tumors depends on continuous exposure to anticancer agents for prolonged periods. Etoposide has a short biological half-life (3.6 hours), and although intraperitoneal injection would result in initial high local tumor concentrations, prolonged exposure of tumor cells may not be possible. It is envisaged that intraperitoneal delivery of etoposide through lipid nanoparticles would be a better approach for effective treatment of peritoneal tumors.

The purpose of present study was to evaluate the efficacy of etoposide-loaded nanoparticle formulations compared with free etoposide in the treatment of Dalton's lymphoma ascites grown in the peritoneal cavity of Balb/c mice, after single-dose intraperitoneal administration. Nanoparticles were prepared from glyceride lipids by melt emulsification-high pressure homogenization technique. Etoposide and its nanoparticle formulations were administered intraperitoneally, and the cell cycle perturbation, cytogenetic damage, cell death (apoptosis), tumor regression, and animal survival were investigated as parameters of response. The results clearly demonstrate that single-dose administration of etoposide-loaded nanoparticle formulations is significantly more effective in the treatment of Dalton's lymphoma ascites as compared with free etoposide.

Materials and Methods

Chemicals

Etoposide was a kind gift obtained from Dabur research center (Mumbai, India) and Cipla Ltd (Mumbai, India). Glycerol monostearate (GMS), glycerol distearate (GDS), and tripalmitin (TP) were purchased from Sisco Laboratories (Mumbai, India). Sodium tauroglycocholate was purchased from Qualigens (Mumbai, India). Hydrogenated soya phosphatidyl choline (HSPC) was purchased from Lipoid (GMBH, Ludwigshafen, Germany). Poloxamer 407 was purchased from BASF (Ludwigshafen, Germany). Propidium iodide (PI), ribonuclease-A (RNase-A), and Hoechst-33258 (bis benzimide (2′-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1H-benzimidazole) trihydrochloride were obtained from Sigma Chemical Co (St Louis, MO). All other chemicals were of analytical grade from BDH, Glaxo laboratories (Qualigens), SRL, and E-Merck (Mumbai, India).

Preparation of Lipid Nanoparticles

The glyceride lipids selected for the preparation of nanoparticles were glycerol monostearate (monoglyceride), glycerol distearate (diglyceride), and tripalmitin (triglyceride). The melting points of GMS, GDS, and TP are 56°C, 55°C, and 82°C, respectively. Lipid nanoparticles were prepared by slight modification of melt emulsification and homogenization technique reported earlier.[15,16] In brief, etoposide was dissolved in a small quantity of methanol (0.2 mL), to it was added HSPC, and the mixture was warmed slightly to form a clear melt. Methanol was then evaporated completely by heating the phase to 55°C. The drug-HSPC melt was added to glyceride lipid(s) and heated to obtain a clear melt. The melt was then heated to 5°C above the melting point of glyceride lipid(s) and emulsified using a blade-type stirrer (Remi, Mumbai, India) at 2000 rpm into the aqueous phase containing 3% wt/vol sodium tauroglycocholate, which was preheated to 5°C above the temperature of the lipid phase and the stirring was continued for 2 minutes. The hot emulsion was then homogenized at a pressure of 10 000 psi for 3 cycles in the high pressure homogenizer (Emulsiflex C5, Avestin, Ottawa, Ontario, Canada) maintained in water bath at 90°C. The nanodispersion formed was spray-dried after the addition of 2 parts by weight lactose monohydrate with respect to the total lipid content in the formulation. The ratio of glyceride lipid(s) to HSPC was 4:1, and that of drug-lipid (including HSPC) was 1:29 for all the nanoparticle formulations.

Characterization of Lipid Nanoparticles

Particle size analysis

The size analysis of nanoparticles was performed by laser diffraction technique using Malvern Hydro 2000SM particle size analyzer (Malvern Instruments, Malvern, UK). The aqueous nanoparticulate dispersion was added to the sample dispersion unit containing stirrer and stirred in order to minimize the interparticle interactions, and the laser obscuration range was maintained between 10% and 20%. The analysis was performed thrice and the average values were taken.

Drug content determination in nanoparticles

Nanoparticle powder (25 mg) was dissolved in a mixture of methanol and chloroform (50:50). Required dilutions were performed with the same solvent mixture and analyzed in a UV-Visible spectrophotometer at 286 nm against the solvent

blank containing the same concentration of HSPC used in the formulation.

Mice and Tumor Transplants

The inbred Balb/c mice (10-12 weeks) used in these studies were obtained from the Institute's central animal facility and weighed 20 to 25 g at the time of tumor induction. They were provided with water and standard mouse food (Liptin, Delhi, India) ad libitum. Dalton's lymphoma cells were maintained by serial passage of tumor cell suspension in the peritoneal cavity of Balb/c mice. All experiments were conducted according to the guidelines established by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and the Indian National Science Academy (INSA) after obtaining permission from the institute's animal ethics committee.

The tumors were implanted by intraperitoneal injection of 5×10^6 Dalton's lymphoma cells (in 0.1-0.15 mL volume) into the peritoneum of Balb/c mice. On day 6 after tumor implantation, the mice gained sufficient tumor growth and were used for further studies.

Administration of Etoposide and Its Nanoparticle Formulations

Tumor bearing mice were held in restrainers for intraperitoneal administration of etoposide and its nanoparticle formulations in ascites bearing mice. Each group consisted of 5 mice. The drug treatment groups were administered with etoposide or etoposide-loaded nanoparticle formulations equivalent to 30 mg/kg etoposide. Etoposide was dissolved in 20% aqueous dimethyl sulfoxide (DMSO) solution. The nanoparticulate dispersions were obtained by dispersing the powder nanoparticles in normal saline. The injection volumes of these preparations were 0.1 to 0.15 mL.

Follow-up

The weights of ascites bearing mice (treated and untreated) were measured on alternate days. Animals with complete tumor regression were observed for their general condition including body weight till death.

Cells were removed from the peritoneal cavity at different times following treatment, washed, and fixed either in 70% ethanol (for flow cytometry) or in acetic acid:methanol (1:3) for morphological analysis.

Cell Cycle Perturbation

Flow-cytometric measurements of cellular DNA content were performed with the ethanol (70%) fixed cells using the intercalating DNA fluorochrome, PI as described earlier.[17] In brief, the cells (~0.5-1 million) were washed in phosphate buffered saline (PBS) after removing ethanol and treated with extraction buffer.[18] Cells were then treated with ribonuclease-A (200 μg/mL) for 30 minutes at 37°C. Subsequently, cells were stained with PI (50 μg/mL) in PBS. Measurements were made with a laser-based (488 nm) flow cytometer (FACS Calibur; Becton, Dickinson and Company, Franklin Likes, New Jersey) and data acquired using the Cell Quest software (Becton, Dickinson and Company). Histograms were analyzed using the Modfit program. Off-line gating using appropriate windows created with untreated cells performed analysis of light scatter.

Micronuclei Formation

Air-dried slides containing acetic acid-methanol (1:3 vol/vol) fixed cells were stained with H-333258 (10 μg/mL in citric acid [0.01 M], disodium phosphate [0.45 M] buffer containing 0.05% Tween-20 detergent) as described earlier.[19] Slides were examined under fluorescence microscope using UV excitation filter and fluorescing nuclei were visualized using a blue emission filter.

Cells containing micronuclei were counted from >1000 cells by employing the criteria of Countryman and Heddle.[20] The fraction of cells containing micronuclei, called the M-fraction (%), was calculated by: M-fraction (%) = $N_m/N_t \times 100$, where N_m is the number of cells with micronuclei and N_t is the total number of cells analyzed.

Apoptosis

Cells undergoing apoptosis were analyzed by morphological examination of cells stained with the DNA specific fluorochrome Hoechst-33258 (similar to micronuclei analysis) as well as by flow-cytometric analysis of DNA content and light scatter. Morphologically, marked condensation and marginalization of chromatin, fragmentation of nuclei and cell shrinkage characterize apoptotic cells, and a good correlation between these morphological changes and DNA ladder (one of the hallmarks of cells undergoing apoptosis) has been demonstrated.[21,22] At least 1000 cells were counted and the percentage apoptotic cells determined from slides prepared as described for micronuclei formation.

In flow-cytometric DNA analysis, the presence of hypodiploid (sub G_0/G_1) population (with PI-stained cells, as described for cell cycle analysis) is indicative of the apoptotic cell population. Cells undergoing apoptosis generally shrink and also show changes in internal structure, which is reflected in the alterations of light scatter. Therefore, treatment-induced changes in forward and side scatter of incident light as well as DNA contents were investigated.

Statistical Analysis

Statistical comparisons of the experimental results were performed by Student *t* test and analysis of variance (ANOVA) at significance level of 0.01 and 0.001.

RESULTS

All 3 types of glyceride nanoparticle formulations (etoposide-loaded tripalmitin [ETP], etoposide-loaded glycerol monostearate [EGMS], and etoposide-loaded glycerol distearate [EGDS]) exhibited more than 97% entrapment efficiency. The nanoparticles were found to be spherical in shape, and the mean diameters of the nanoparticles are shown in Table 1.

Tumor Response: Animal Survival

Each treated and untreated group of animals consisted of 5 mice. The tumor weight was estimated by subtracting the initial weight of mice from the final weight ($W_t - W_0$) and plotted against number of days. The tumor burden of mice treated with etoposide and nanoparticle formulations was greatly reduced than the W_0 up to 4 to 6 days, followed by an increase at later time intervals (Figure 1). The increase in tumor burden of placebo (drug-free nanoparticles) and DMSO-treated groups was marginally less than that of untreated mice. At the end of 3 weeks of follow-up (30 days) following various treatments, it was clear that all the nanoparticle formulations had a profound effect on the tumor progression. While the relative tumor burden with etoposide alone was 60% of the untreated mice, it was less than 10% to 30% with different formulations (Figure 1). The rate of tumor regrowth following administration of free etoposide was consistently higher than that observed with all 3 nanoparticle formulations and was in the following order ETP < EGMS < EGDS < etoposide indicating the greater effectiveness of nanoparticle formulations compared with free etoposide in tumor control, which demonstrated lowest tumor burden in case of ETP and EGMS nanoparticles. The higher tumor regression also resulted in a significant increase in the duration of survival of the tumor bearing mice. The survival of mice increased when treated with ETP

Table 1. Entrapment Efficiencies and Mean Particle Diameter of Different Etoposide-loaded Lipid Nanoparticle Formulations*

Formulation Type	Entrapment Efficiency (%)	Mean Particle Diameter (nm)
EGMS	97.63	383.0
EGDS	96.82	387.0
ETP	99.40	354.0

*EGMS indicates etoposide-loaded glycerol monostearate nanoparticles; EGDS, etoposide-loaded glycerol distearate nanoparticles; and ETP, etoposide-loaded tripalmitin nanoparticles. The results are the average of 3 experiments.

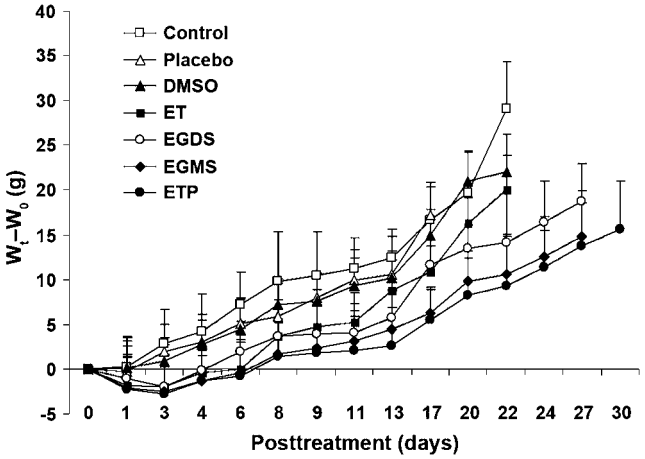

Figure 1. Effects of intraperitoneally administered etoposide and etoposide-loaded nanoparticle formulations on tumor growth in Dalton's lymphoma bearing Balb/c mice. Each treated and untreated group consisted of 5 mice (n = 5). The values are mean ± SD. DMSO, dimethyl sulfoxide; ET, etoposide; EGMS, etoposide-loaded glycerol monostearate nanoparticles; EGDS, etoposide-loaded glycerol distearate nanoparticles; and ETP, etoposide-loaded tripalmitin nanoparticles.

nanoparticle formulation followed by EGMS (27.3%) and EGDS (27.3%) nanoparticle formulations, which was found to be significantly higher ($P < .001$) compared with the mice treated with etoposide (Figure 1). Since this decrease in tumor burden could arise from cytostatic effects (delayed/arrest of cell progression) as well as cytotoxic effects (cell death), we investigated these factors at regular time intervals in cells obtained from the peritoneal cavity. Tumor growth and mice survival were studied as a parameter of tumor response following various treatments. The tumor burden was determined by monitoring mice weight at regular time intervals.

Cell Cycle Perturbation

Dalton's lymphoma a tetraploid clone can be easily distinguished from the diploid by the DNA content. Figure 2 shows the cell cycle distribution of Dalton's lymphoma tumor cells collected from the peritoneum of mice at different posttreatment time intervals. A time-dependent increase in the tumor burden (cell numbers) in untreated tumor cells is evident from the decreased normal cell population up to 17 days after treatment. Treatment with placebo nanoparticles and DMSO (20%) showed the cell cycle distribution similar to that of control mice (figure not shown). One day after treatment with etoposide, the cells were arrested in G_2 (65%) (Figure 3) phase of cell cycle together a hypodiploid peak of cells, indicating the cells undergoing apoptosis after treatment (Figure 2F). On day 3 after etoposide treatment, the hypodiploid peak as well as G_2 arrest increased further (G_2 = 73%). The percentage of dead cells also increased at this time point. After 8

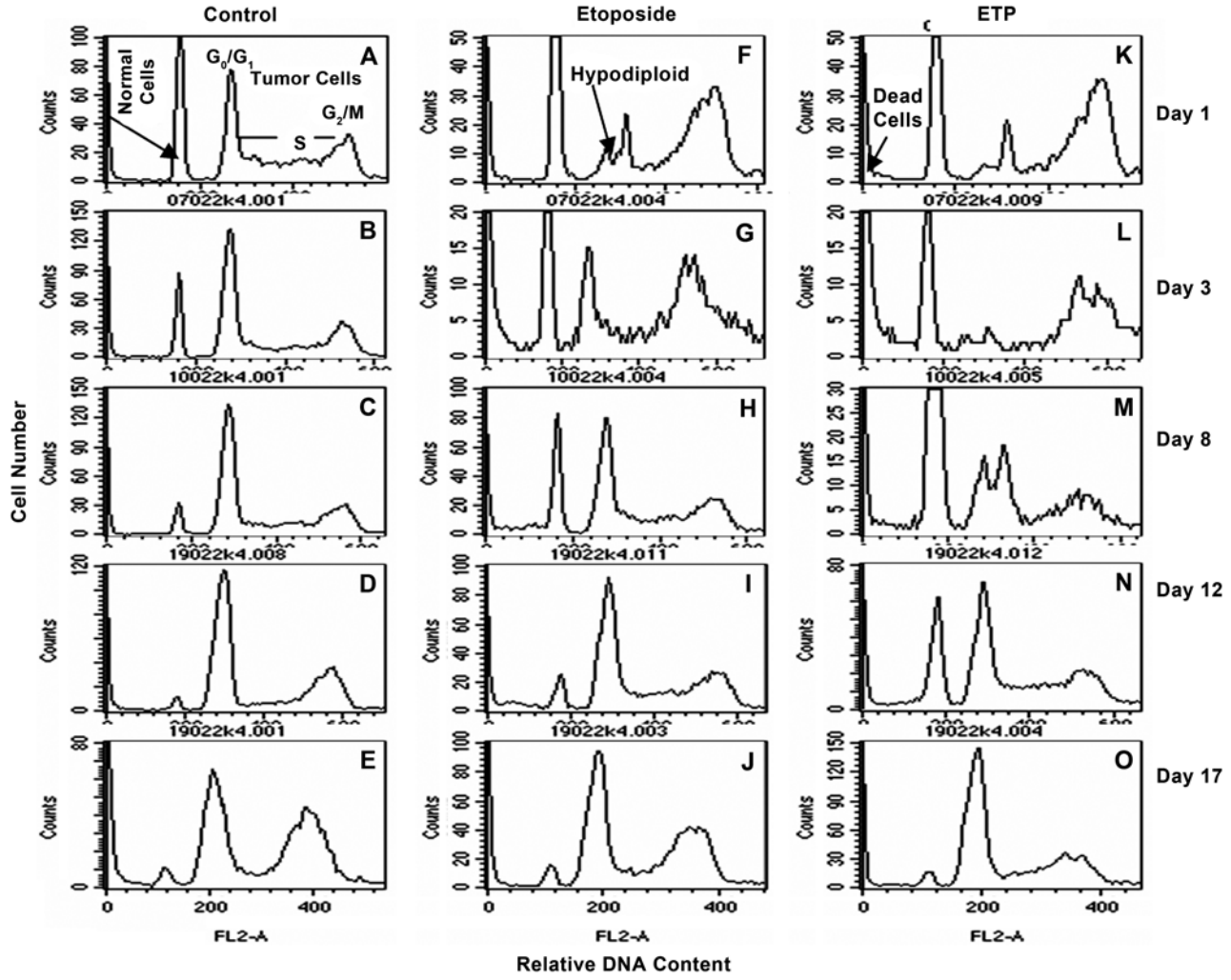

Figure 2. Effects of intraperitoneally administered etoposide and etoposide-loaded lipid nanoparticle formulation (ETP) on the cell cycle perturbations of cells collected from the Dalton's lymphoma tumor bearing Balb/C mice. Columns 1, 2, and 3 indicate the flow-cytometric curves of cells collected from the peritoneal cavity of control (untreated mice), etoposide-treated mice, and etoposide-loaded tripalmitin (ETP) nanoparticle formulation-treated mice, respectively.

days posttreatment, a progressive repopulation of tumor cells was found up to day 17 of observation (Figure 2, middle panel). Enhanced G_2 arrest, increase in the number of dead cells, as well as high percentage of cells with a hypodiploid peak could be observed in mice treated with nanoparticle formulations as early as 1 day posttreatment. On day 3, S phase cells (complying tumor cell proliferation) greatly reduced in case of both free etoposide and nanoparticle treatment (Figure 2G, L). However, the frequency of dead cells was found to be higher in cells obtained from animals treated with all nanoparticle formulations when compared with free etoposide (Figure 2 data shown only for ETP formulation). Day 3 posttreatment also showed higher apoptosis (Figure 4) as indicated by the higher number of dead cells (Figure 2) in both etoposide and nanoparticle formulations. The proliferative cell (S phase) is still observed less in these groups. On day 8 posttreatment, the free etoposide showed a greater decrease of apoptosis as indicated by the reduced number of dead cells vis-a-vis recovery of the tumor cells in the cell cycle. The frequency of dead cells treated with the formulations remained high till this time. EGDS and ETP nanoparticle-treated cells still exhibited hypodiploid peaks and less S-phase cells (8 days). On day 12, the frequency of cells in the S phase increased significantly ($P < .001$) indicating active cell repair process, while the G_2/M phase cells reduced owing to their recovery from the treatment-induced damage and back to their normal course of cell cycle, or they may have undergone apoptosis. On day 17, the cell cycle seemed to be recovered from the death processes (apoptosis). Increased number of dead cells in etoposide and its formulation on day 1 to day 3 posttreatment (Figure 2 F, G, K, L), followed by time-dependent decreased cell death (Figure 2 H, I, J, M, N, O) could be either mitotic cell death or an apoptotic death followed by secondary necrosis. Therefore, induction of micronuclei as well as apoptosis was also studied in these samples.

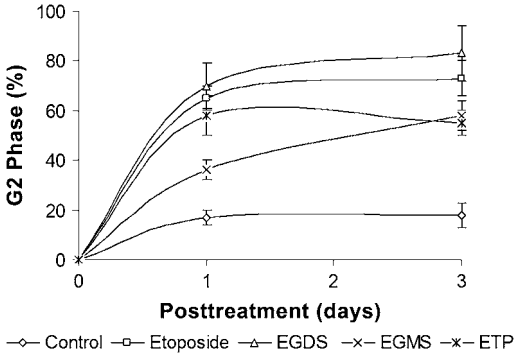

Figure 3. Effects of intraperitoneally administered etoposide and its lipid nanoparticle formulations on cell cycle (G_2 phase) arrest in Dalton's lymphoma tumor cells obtained from the peritoneal cavity of mice treated by intraperitoneal injection. Each treated and untreated group consisted of 5 mice (n = 5). The error bars indicate the SD values. EGMS, etoposide-loaded glycerol monostearate nanoparticles; EGDS, etoposide-loaded glycerol distearate nanoparticles; and ETP, etoposide-loaded tripalmitin nanoparticles.

Cytogenetic Damage

The sensitivity of cells against antitumoral drugs could be determined by the mitotic and interphase (apoptotic) death. The induction of mitotic death was studied by investigating the effects of etoposide and its nanoparticle formulations on induction of micronuclei expression (MN) in Dalton's lymphoma tumor cells. Morphological analysis of cells obtained from ascitic fluid during follow-up clearly showed that the nanoparticle formulations including free etoposide have a marginal increase in the number of micronuclei induction than the controls on day 3 after treatment (1%-4%) (Table 2).

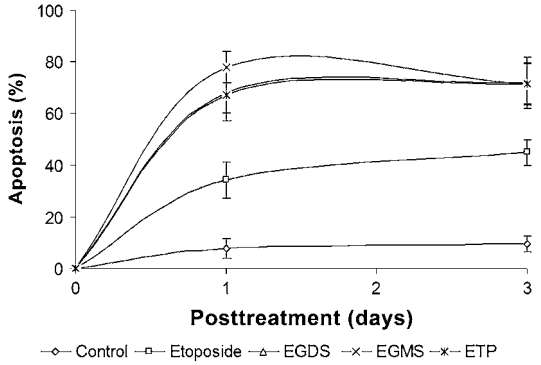

Figure 4. Effect of intraperitoneally administered etoposide and etoposide-loaded lipid nanoparticle formulations on induction of apoptotic cell death (morphological analysis) in Dalton's lymphoma cells obtained from the peritoneal cavity of mice treated by intraperitoneal injection. Each treated and untreated group consisted of 5 mice (n = 5). The error bars indicate the SD values. EGMS, etoposide-loaded glycerol monostearate nanoparticles; EGDS, etoposide-loaded glycerol distearate nanoparticles; and ETP, etoposide-loaded tripalmitin nanoparticles.

Table 2. Effects of Etoposide and Its Lipid Nanoparticle Formulation on Induction of Micronuclei in Dalton's Lymphoma Cells Obtained From Peritoneal Cavity of Balb/c Mice at Various Time Intervals (morphological observation)*

Posttreatment Time	Groups	Micronuclei (%)† ± SD
Day-1	Control	0.10 ± 0.40
	Etoposide	1.40 ± 0.26
	ETP	1.50 ± 0.38
Day-3	Control	0.60 ± 0.21
	Etoposide	3.77 ± 0.51
	ETP	2.50 ± 0.43
Day-8	Control	0.70 ± 0.24
	Etoposide	0.40 ± 0.36
	ETP	0.35 ± 0.48

*ETP indicates etoposide-loaded tripalmitin nanoparticles. Data are shown for only one formulation because similar frequencies of micronuclei were observed in other formulation. ETP indicates etoposide-loaded tripalmitin nanoparticles.
†The differences in the micronuclei observed between the groups in each day, or between different days, were not significant ($P > .5$).

However, at later time intervals (more than 3 days), the fraction of micronucleated cells decreased considerably, and the values were not significant from the values of untreated samples. These observations indicate that the death of Dalton's lymphoma tumor cells caused by etoposide and its formulation was primarily not mitotic. Therefore, samples were evaluated for apoptotic mode of death processes. Since interphase (apoptotic) death also contributes to the cytotoxicity of etoposide, we evaluated apoptotic mode of death processes beside mitotic death following various treatments.

Apoptosis

Since apoptosis contributes to the antitumoral drug-induced loss of clonogenicity besides mitotic death, we investigated the effects of etoposide and its nanoparticle formulation-induced apoptosis in Dalton's lymphoma cells. The Dalton's lymphoma cells collected from the ascites fluids of peritoneum of mice at different posttreatment time intervals were processed for both morphological as well as flow-cytometric analysis to evaluate the interphase mode of cell death (apoptosis). All etoposide-loaded nanoparticle formulations showed high apoptosis, starting from day 1 to day 17 compared with free etoposide. The fraction of apoptotic cells slowly reduced with EGMS after day 3 and EGDS nanoparticles after day 8. The frequency of apoptotic cells reached a plateau after 8 days in case of etoposide and EGMS nanoparticle-treated mice, and after 12 days in case of EGDS nanoparticles. ETP nanoparticles exhibited higher apoptosis than all other formulations. Mice treated with ETP resulted in 59% apoptosis, which is very high compared with free

etoposide (23% apoptosis) on day 17 posttreatment. The apoptotic frequency in cells after 17 days posttreatment was in the following order: ETP > EGMS > EGDS > etoposide. On the other hand, control (untreated), placebo nanoparticles, and DMSO treatment did not exhibit marked apoptosis. The fraction of apoptotic cells in all these 3 groups was almost similar. Flow-cytometric analyses correlated well with the results obtained by morphological analysis. Treatment induced the time-dependent significant increase in hypodiploid peak (indicative of cells undergoing apoptosis) in etoposide up to 3 days and up to 8 days in case of nanoparticle formulations (Figure 2 K, L, M).

DISCUSSION

Tumor response following a single systemic administration of the drug-like etoposide in mice is determined by the in vivo half-life, cellular residence time of the drug, and the nature of cellular responses elicited by the drug-induced lesions. One of the drawbacks associated with intraperitoneal chemotherapy using free drugs is their rapid disposition from peritoneal cavity and distribution to other tissues. Hence the local drug concentrations required to elicit an effective chemotherapeutic response may not be available in the peritoneal cavity. To be effective in peritoneal carcinomas, an antitumor drug should show low peritoneal clearance.[23] The local chemotherapeutic response may be improved by incorporation of anticancer agents into a delivery system that can facilitate sustained drug release by remaining in the peritoneal cavity for sufficient time periods. Long-term intraperitoneal administration of low dose etoposide is reported to be clinically useful for postoperative and maintenance chemotherapy.[24] Incorporation of etoposide in Lipiodol (an oil) and its intraperitoneal administration greatly reduced its disposition into blood and delivered more etoposide for a longer time to the omentum (intraperitoneal tissue). This preparation also increased significantly the survival time of mice implanted intraperitoneally with P388 leukemia cells, compared with etoposide solution.[25,26] The intraperitoneally delivered etoposide, using totally implantable peritoneal access system, was reported to be beneficial for the treatment of advanced gastric cancer with peritoneal metastasis.[27] Etoposide is an anticancer agent with apoptosis induction property and is reported to be effective in the treatment of lymphomas.[12] In this study, etoposide was incorporated into lipid nanoparticles made from different glyceride lipids, and the effects of etoposide-loaded nanoparticle formulations and free etoposide on the Dalton's lymphoma ascites tumor were studied with respect to cell cycle perturbations, cytogenetic damage, and apoptosis.

Morphological analysis of the cells collected from treated mice bearing Dalton's lymphoma peritoneal tumor indicates a marginal increase in micronuclei formation in case of treatment with etoposide and its nanoparticle formulations compared with control, indicating that the cell death is not predominantly owing to the induction of mitotic death. Study of apoptosis indicated that the etoposide and its nanoparticle formulations induced high apoptosis in the cells. The characteristic apoptotic properties included cell shrinkage, nuclear chromatin condensation and fragmentation, and cytoplasmic budding. The apoptosis induction was higher with etoposide-loaded nanoparticle formulations, which may be attributed to their effective cellular penetration and probably sustained drug release inside the cells. Among all the formulations, ETP nanoparticles showed higher apoptosis, which may be due to the relatively slower drug release inside the cells and effective cellular retention. Another possibility is the enhancement in the drug-intracellular target interaction by the nanoparticle encapsulated etoposide owing to the endocytosis, sustained release, and greater tumor retention properties of nanoparticles.

The tumor growth follow-up was done on Balb/c mice of 10 to 12 weeks old. One control group of healthy mice (3 mice) was observed for growth (increase in weight owing to age and food) along with the treated groups. No significant weight increase in mice was observed as generally the Balb/c mice do not show significant weight gain after this age. Hence, the weight gain of the mice was not considered. Estimation of tumor burden by subtracting the initial weight of mice from the final weight ($W_t - W_0$) would have a possibility of contamination of the resultant weight by drug-induced weight loss. Hence, a group of control healthy mice injected with etoposide (30 mg/kg) was checked for any possible decrease in weight as a result of the toxicity. The decrease in weight for the administered dose was very low (not more than 0.3 gm per mouse) during the treatment period and was not significant to subtract from the weights of the treated mice.

Tumor progression in the control and placebo-treated animals is evident from Figure 1. Treatment with either placebo or DMSO solution did not result in significant changes in the cell cycle distribution. Treatment with etoposide or its nanoparticle formulations resulted in significant changes in the cell cycle distribution such as arrest of cells in G_2 phase together with hypodiploid peak characterizing apoptotic cells and the reduced S phase. The G_2 arrest resulted in increase in the dead cell number. However, after 3 days posttreatment, repopulation of cells occurred in the mice treated with etoposide (Figure 2). This may be to the result of the rapid clearance of etoposide from the peritoneal cavity compared with the nanoparticle formulations. The effectiveness of nanoparticle formulations was evident from the high frequency of dead cells observed even after 8 days, which was not the case with etoposide. The EGDS and ETP nanoparticles still showed characteristic hypodiploid peaks and reduced S phase, indicating the prolonged apoptotic

induction in cells. The hypodiploid peak in case of treatment with etoposide was not found after 3 days and was prominent in the treatment with nanoparticle formulations, indicating the effective role of nanoparticle formulations in the apoptotic induction. However, the shortened S phase was not observed in case of etoposide treatment after 3 days but was prominent in case of the treatment with nanoparticle formulations, indicating prolonged effect of these formulations on the cell cycle. Among the 3 lipid nanoparticle formulations, the frequency of dead cells was more even after 8 days with ETP nanoparticle treatment, indicating the greater efficacy of this formulation in apoptotic induction. The results reveal that the nanoparticles exhibit sustained drug release properties, which are evident from the continuous apoptosis seen with etoposide-loaded nanoparticle formulations when compared with free etoposide. The latter decrease in apoptosis in case of nanoparticle formulations can be attributed to the dilution effects of nanoparticles or to drug depletion resulting in repopulation of cells, which is linked to proliferation.

In general, after intraperitoneal administration, the nanoparticles rapidly escape from the peritoneal cavity and distribute to other sites such as lymphatic system. Poly(lactide-co-glycolide) nanoparticles (<1 μm) have been shown to enhance the lymphatic transport of aclarubicin after intraperitoneal injection.[28] However, the prolonged effect of nanoparticle formulations observed in our case probably indicates the retention of majority of the administered nanoparticles in the peritoneal cavity for prolonged time, facilitating sustained drug release.

The initial decrease in tumor burden in mice treated with etoposide and its nanoparticle formulations is the result of the rapid apoptosis and cytogenetic damage, which decreased with time resulting in repopulation of cells. Increase in tumor burden in case of nanoparticle formulations after 4 to 6 days is the result of the decrease in the amount of drug available for the cells to cause cellular damage, which can be due either to decrease in nanoparticle content in the peritoneum (dilution effect of nanoparticles) or completion of drug release from the nanoparticles. The absence of apoptosis and continuous tumor progression in the case of treatment with placebo nanoparticles indicates that the placebo nanoparticle matrix does not have any effect on the control of tumor cell population. The apoptotic death can be to the result of the induction of DNA lesions as well as oxidative stress and other effects such as ligand-receptor (cell membrane) interaction. DMSO (20% aqueous solution, the administration vehicle) was also used as one of the treatment groups in order to confirm that the DMSO has no significant influence on the tumor inhibition. The results clearly demonstrated no effect of this compound on the tumor inhibition properties. The administration of ETP nanoparticles inhibited the tumor growth for a long period of time. In vitro studies demonstrated that all 3 types of nanoparticle formulations exhibited sustained release of etoposide, after a slight initial burst corresponding to the surface adsorbed drug, (t_{50}, time taken for 50% drug release values, were calculated as 30.5, 35, and 46.75 hours for EGMS, EGDS, and ETP, respectively).[29] The drug release was in the order of ETP < EGDS < EGMS. Thus the more sustained effects of ETP nanoparticles were probably owing to their longer residence time and prolonged drug release properties (compared with the EGDS and EGMS nanoparticles). The experimental results indicated that among the 3 nanoparticle formulations studied, the ETP nanoparticles showed greater and more prolonged apoptotic induction properties, resulting in the higher increase in survival time of tumor bearing mice. Hence, this nanoparticle formulation may be a promising candidate for clinical trials for the treatment of peritoneal carcinomas and for local chemotherapy.

Conclusion

Intraperitoneal administration of etoposide and its lipid nanoparticle formulations resulted in rapid induction of apoptosis in cells in mice bearing Dalton's lymphoma in ascites form. The placebo nanoparticle formulation and DMSO solution used to dissolve etoposide did not show any effect on the cell cycle, cytogenetic damage, or survival time of tumor bearing mice. The results of morphological studies indicated that cell death as a result of the treatment is mainly owing to apoptosis and not mitotic death. Etoposide-loaded nanoparticle formulations showed significantly higher apoptosis induction ($P < .001$) for a more prolonged period and higher increase in survival time of tumor bearing mice, when compared with free etoposide. Among the 3 nanoparticle formulations, ETP nanoparticle formulation showed significantly higher apoptosis and increase in survival time of tumor bearing mice. Our study indicates that the ETP nanoparticle formulation may be a promising delivery system for the effective treatment of ETP-sensitive peritoneal carcinomas and peritoneal metastasis.

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